STORE OPERATED Ca^{2+} CHANNELS IN LIVER CELLS: REGULATION BY BILE ACIDS AND A SUB-REGION OF THE ENDOPLASMIC RETICULUM

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SUMMARY

Cholestasis is an important liver pathology. During cholestasis bile acids accumulate in the bile canaliculus affecting hepatocyte viability. The actions of bile acids require changes in the release of Ca^{2+} from intracellular stores and in Ca^{2+} entry. The target(s) of the Ca^{2+} entry pathway affected by bile acids is, however, not known. The overall objective of the work described in this thesis was to elucidate the target(s) and mechanism(s) of bile acids-induced modulation of hepatocytes calcium homeostasis.

First, it was shown that a 12 h pre-incubation with cholestatic bile acids (to mimic cholestasis conditions) induced the inhibition of Ca^{2+} entry through store-operated Ca^{2+} channels (SOCs), while the addition of choleretic bile acids to the incubation medium caused the reversible activation of Ca^{2+} entry through SOCs. Moreover, it was shown that incubation of liver cells with choleretic bile acids counteracts the inhibition of Ca^{2+} entry caused by pre-incubation with cholestatic bile acids. Thus, it was concluded that SOCs are the target of bile acids action in liver cells.

Surprisingly, despite the effect of choleretic bile acids in activating SOCs, the Ca^{2+} dye fura-2 failed to detect choleretic bile acid-induced Ca^{2+} release from intracellular stores in the absence of extracellular Ca^{2+}. However, under the same conditions, when the sub-plasma membrane Ca^{2+} levels were measured using FFP-18 Ca^{2+} dye, choleretic bile acid induced a transient increase in FFP-18 fluorescence. This evidence suggested that choleretic bile acids-induced activation of Ca^{2+} entry through SOCs, involving the release of Ca^{2+} from a region of the endoplasmic reticulum (ER) located in the vicinity of the plasma membrane.

To explore the possible requirement of a small sub-region of the ER in SOCs activation, TRPV1 channels ectopically expressed in the ER, were used as an alternative approach. Using this strategy it was demonstrated that TRPV1 agonists mediated the release of Ca^{2+} from the bulk of the ER (detected by cytoplasmic fura-2 but not by sub-plasma membrane FFP-18) but failed to activate SOCs in liver cells. This represents additional evidence for the suggested role of a small sub-region of the ER in the activation of SOCs.

Finally, the effect of either choleretic bile acid taurodeoxycholate (TDCA) or TRPV1 agonists, on the distribution of the ER calcium sensor stromal interaction molecule 1 (STIM1) (required for SOCs activation) was investigated. It was observed that TDCA caused the reversible redistribution of STIM1 into punctate aggregates within the ER sub-region in the vicinity of the plasma membrane, similar to that induced by the ER (Ca^{2+}+Mg^{2+}) ATPase pump inhibitor, thapsigargin. TRPV1 agonists however, do not induce the redistribution of STIM1 proteins in the majority of cells expressing TRPV1.

In summary, the work described in this thesis indicates that the main type of plasma membrane Ca^{2+} channel, inhibited by cholestatic bile acids and activated by choleretic bile acids, is the hepatocyte SOC. Additionally, this work provides evidence that bile acids activated SOCs by a mechanism that involves the release of Ca^{2+} from- and the STIM1 redistribution within- a small sub-region of the ER located in the vecinity of the plasma membrane.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Joel Castro Kraftchenko

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ABBREVIATIONS

$[Ca^{2+}]_{cyt}$	Cytoplasmic free Ca^{2+} concentration
$(Ca^{2+})_{SPM}$	Sub-plasma membrane Ca^{2+} levels
$(Ca^{2+}{}_{ext})$	Extracellular Ca^{2+}
CA	Cholic acid
CRAC	Ca^{2+} Release-Activated Ca^{2+} Channel
DBHQ	Di-*tert*-butylhydroquinone
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulfoside
EGTA	Ethylene Glycol-bis(b-aminoethyl ether)-*N,N,N´N´*-Tetraacetic Acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FCCP	Carbonylcyanide-p-trifluoromethoxyphenylhydrazone
Gd^{3+}	Gadolinium
GFP	Green Fluorescent Protein
I_{crac}	Ca^{2+} Release-Activated Ca^{2+} Current
IP_3	Inositol 1,4,5-trisphosphate
IP_3Rs	Inositol 1,4,5-trisphosphate receptors
KRH	Krebs-Ringer-HEPES buffer
La^{3+}	Lanthanum
LCA	Lithocholic acid
PBS	Phosphate Buffered Saline
PM	Plasma membrane
PMCA	Plasma Membrane (Ca^{2+} + Mg^{2+}) ATP-ases pump

ROI Regions of interest

RR Ruthenium red

RTX Resiniferatoxin

SERCA Endoplasmic Reticulum (Ca^{2+} + Mg^{2+}) ATP-ase pump

SOCs Store-Operated Ca^{2+} channels

STIM1 Stromal Interaction Molecule 1

TCA Taurocholic acid

TDCA Taurodeoxycholic acid

Tg Thapsigargin

TLCA Taurolithocholic acid

TMRM Tetramethyl Rhodamine Methyl ester

TPEN *N,N,N',N'*-tetrakis(2-pyridylmethyl) ethylene diamine

TRPV1 Transient Receptor Potential cation channel, subfamily V, member 1

TUDCA Tauroursodeoxycholic acid

UDCA Ursodeoxycholic

YFP Yelow Fluorescent Protein

2-APB 2-aminoethoxydiphenylborane

CHAPTER I: INTRODUCTION

The work undertaken in this thesis investigates the effects of bile acids on the regulation of Store-Operated Ca^{2+} Channels (SOCs) in liver cells. Additionally, the role of the endoplasmic reticulum in the activation of SOCs will also be examined. Therefore this introductory chapter will cover: (i) the liver role, physiology and structure, including cellular organisation and function; as well as the causes and consequences of the pathological condition, cholestasis; (ii) the regulation of Ca^{2+} homeostasis in liver cells, including the different Ca^{2+} permeable channels presents in the plasma membrane; (iii) the physiological relevance, properties and activation mechanism of one of these Ca^{2+} permeable channels, called the store-operated Ca^{2+} channels (SOCs); (iv) finally, some facts about the role of the endoplasmic reticulum, in the activation of SOCs, will be presented.

1.1 Liver physiology and effects of bile acids accumulation during liver cholestasis

1.1.1. Roles of the liver in whole body homeostasis

The liver plays a central role in the metabolism, detoxification, and overall regulation of the body homeostasis. During fetal development, the liver is the primary organ of hematopoiesis and is required for the maintenance of normal bone marrow formation. The liver is involve in the efficient uptake of amino acids, carbohydrates, lipids and vitamins and their subsequent storage, metabolic conversion and release into the blood and bile. Additionally, the liver performs

several roles in carbohydrate metabolism, including: (i) the synthesis of glucose from certain amino acids, lactate or glycerol (Gluconeogenesis); (ii) the breakdown of glycogen into glucose (Glycogenolysis); (iii) the formation of glycogen from glucose (Glycogenesis); and (iv) the breakdown of insulin and other hormones. The liver also performs several roles in lipid metabolism including cholesterol synthesis and triglycerides production.

This organ is responsible of the synthesis and trans-cellular movement of bile acids, bile fluid, and the synthesis and secretion of proteins (Leite and Nathanson, 2001; Boyer, 2002b). Additionally, the liver is the hepatic bio-transformation of hydrophobic substances into water-soluble derivates that can be excreted into bile or urine (Leite and Nathanson, 2001). The liver breaks down toxic substances and most medicinal products. Furthermore, the large vascular capacity of the liver operates as a reservoir of blood volume in the circulation (Miayai, 1991). The liver's function also influences the central nervous system, endocrine organs and the immune system (Leite and Nathanson, 2001).

In human, the liver is among the few internal organs capable of regenerating lost tissue; as little as 25% of intact liver can regenerate into a whole liver again. There is no artificial organ, or device, capable of emulating all the functions of the liver.

1.1.2. The structure of the structure

The liver is the largest glandular organ of the body, constituting approximately 2% to 5% of the body weight. According to the "functional vascular anatomy" the liver is divided into VIII segments with independent afferent and efferent blood supplies. The caudate segment (segment I) is considered autonomous and the remaining segments are named clockwise from II to VIII (Dawson and Tan, 1992). The

"classical anatomy" divides the organ in four lobes, according to the peritoneal attachments and surface fissures of the liver (Fig. 1.1 A, a). Within each liver lobe, the tissue (liver parenchyma) is arranged into hexagonal-shaped lobules each comprised of a central vein surrounded by six portal triads (Fig. 1.1 A, b) (Young and J.W, 2000; Mohammed and Khokha, 2005). Each portal triad is comprised of a hepatic vein, hepatic artery and bile duct (Fig. 1.1 A, c).

The predominant cell in the liver is the hepatocyte (parenchymal cell) which represents ~ 70% of all cells in the liver (Dawson and Tan, 1992; Boyer, 2002b; Mohammed and Khokha, 2005). Endothelial cells, biliary epithelial cells (cholangiocytes), hepatic stellate cells, Kupffer cells (macrophages) and oval cells, also play important roles in the liver. Hepatocytes, often considered as specialised epithelial cells, are organised in single-cell plates (Fig. 1.1 B). The hepatocyte plates are perfused by blood from the gut (hepatic portal vein) and heart (hepatic artery), which drains into the central vein (Fig. 1.1 B). The bile canaliculi (surrounded by the adjacent membranes of two hepatocyte lines) collects bile fluid from each hepatocyte and moves this fluid to the bile duct (Figs. 1.1 A and 1.2).

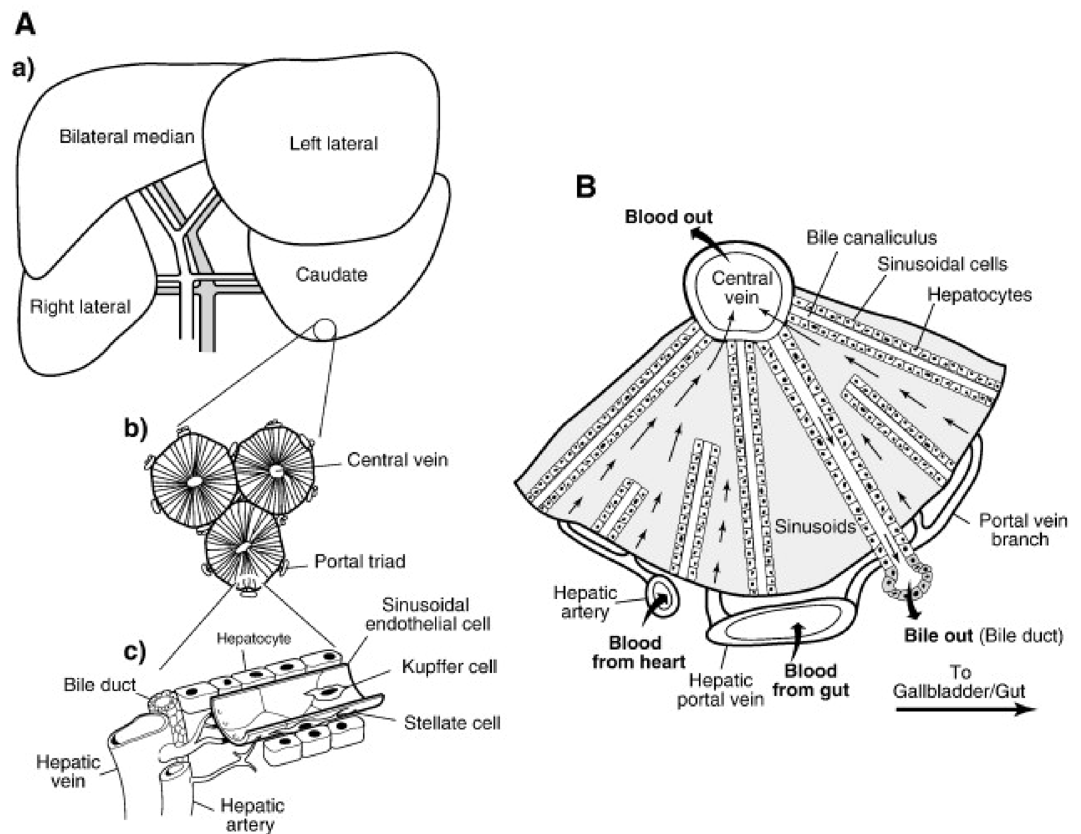

Fig. 1.1. Liver anatomy and hepatocytes organisation. (A) Drawing of the major lobes of rat liver (a), the relationship between the central vein and portal triads (b), and the arrangement of the hepatic vein, hepatic artery, bile duct, hepatocyte plate and sinusoidal space (c), is presented. (B) A schematic drawing of the hepatocyte plates showing the direction of blood flow from the hepatic portal vein and hepatic artery to the central vein, and the direction of bile flow through the bile canaliculus. This figure is adopted from (Barritt *et al.*, 2008).

Consistent with the complex function and architecture of the liver, hepatocytes are highly differentiated cells exhibiting spatial polarisation and a characteristic intracellular organization (Berry *et al.*, 1991; Hubbard *et al.*, 1994; Zegers and Hoekstra, 1998; Boyer, 2002b; Wakabayashi *et al.*, 2006). Some features of the spatial polarity of hepatocytes are schematically shown in Fig. 1.2. The plasma membrane (PM) of each hepatocyte is differentiated into the sinusoidal (basal), contiguous (lateral), and canalicular (apical domains) domains. The sinusoidal domain, facing the blood circulation, comprises more than 70% of the surface area, contains many receptors, transport systems and ion channels, which facilitate exchange of nutrients and other solutes between hepatocytes and the systemic circulation. The lateral domain is mainly involved in intracellular communications through cell adhesion molecules and intracellular junctions such as tight junctions, desmosomes, and gap junctions. Gap junctions permit the movement of molecules between adjacent hepatocytes (Berry *et al.*, 1991; Hubbard *et al.*, 1994; Zegers and Hoekstra, 1998; Boyer, 2002b; Wakabayashi *et al.*, 2006). The canalicular domain (formed by the facing plasma membrane of two adjacent hepatocytes), is separated from the sinusoidal and lateral domains by tight junctions. It has been described that the canalicular domain is involved in the secretion of bile acids and polymeric immunoglobulin A into the bile; the unique excretory products of hepatocytes (Nathanson and Boyer, 1991). The trafficking of proteins and vesicles through the hepatocyte cytoplasmic space is critical for the maintenance of hepatocyte spatial polarity and for many hepatocyte functions (Zegers and Hoekstra, 1998; Wakabayashi *et al.*, 2006).

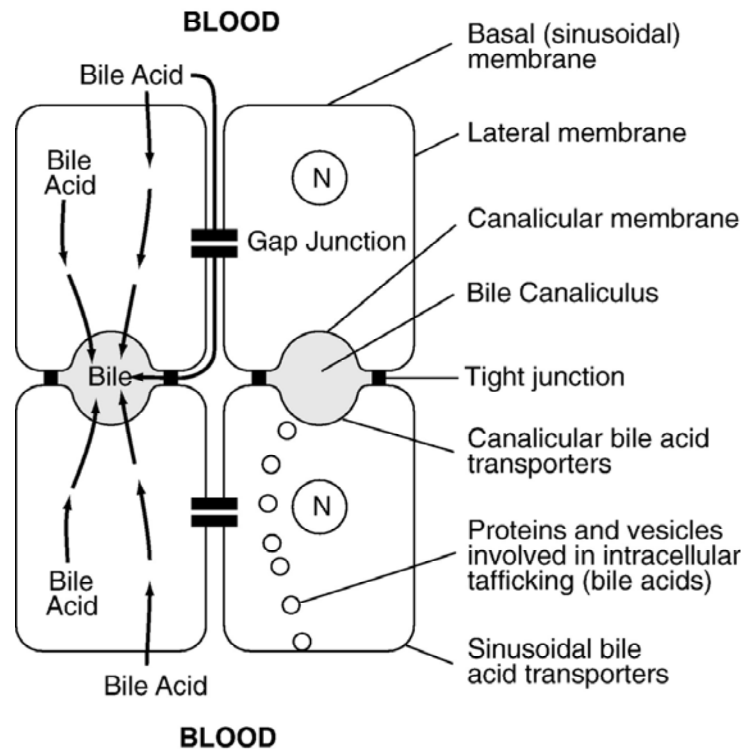

Fig. 1.2. Schematic representation of the hepatocyte spatial polarity. The scheme shows the different hepatocytes domains and the pathways of bile acid movement and vesicle trafficking in hepatocytes within the hepatocyte plate. This figure is adapted from (Barritt *et al.*, 2008).

As shown in Fig. 1.3, hepatocytes are enriched in rough and smooth endoplasmic reticulum (ER). This comprises an extensive array of interconnecting membranes that exhibit remarkable spatial extensions forming cisternae and tubules and has continuity with the nuclear envelope (Wang *et al.*, 2000). The ER is the largest compartment in hepatocytes, its surface area is ~ 38 times that of the PM and constitutes ~ 15% of the total cellular volume (Lippincoott-Schwartz, 1994). As described below, this intracellular organelle is one of the focal points of this thesis.

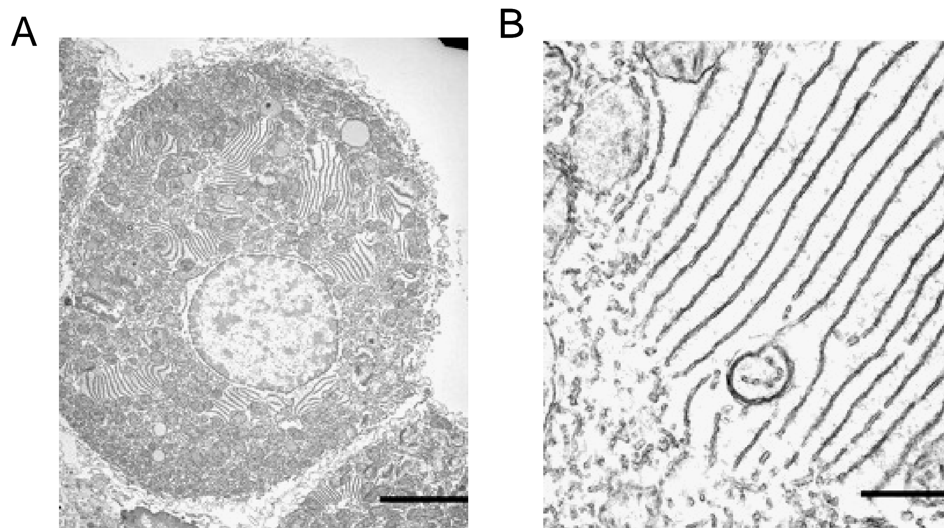

Fig. 1.3. Electron micrographs of hepatocytes. Panel A shows electron micrographs of whole hepatocytes showing different intracellular organelles. Panel B shows a zoom image of an intracellular region showing in fine detail the structure of the endoplasmic reticulum. Scale bars are 5 μm This figure is adapted from (Wang *et al.*, 2000).

1.1.3. Cholestasis and the consequences of bile acid accumulation in liver

Cholestasis is an important liver pathology, which can lead to primary sclerosis and liver failure (Boyer, 2002a; Maddrey, 2002; O'Leary and Pratt, 2007). Cholestasis arises from hepatocyte dysfunction or intrahepatic or extrahepatic biliary obstruction leading to impaired movement of bile along the biliary duct tree and impaired secretion of the components of bile fluid into the bile canaliculus. Cholestasis has many causes including primary sclerosing cholangitis, primary biliary cirrhosis, liver disorders associated with liver injury, and genetic abnormalities in hepatocyte bile acid transporters. Additionally, cholestasis is associated with decreases in the expression and/or activity of hepatocyte bile acid transporters (Boyer, 2001).

In cholestasis, conjugated and unconjugated bile acids accumulate in hepatocytes and the blood (Boyer, 2001). Hepatocytes play a central role in the synthesis of bile acids and in the movement of bile acids from the portal blood to the gallbladder and intestine. Bile acids are transported from the blood across the basal (sinusoidal) and basolateral membranes of hepatocytes, through the cytoplasmic space, and then across the canalicular membrane into the bile canaliculus.

Bile acids can be classified according their hydrophobicity, the more hydrophobic bile acids (such as taurolithocholic (TLCA), lithocholic (LCA), cholic (CA) and taurocholic (TCA) acids), are called cholestatic bile acids; while the less hydrophobic bile acids (such as taurodeoxycholic (TDCA), tauroursodeoxycholic (TUDCA) and ursodeoxycholic (UDCA) acids), are called choleretic bile acids.

It has been shown that cholestatic bile acids induce bile flow inhibition (Boyer, 2002a). Additionally, it has been reported that cholestatic bile acids induce liver injury, leading to apoptosis and necrosis of hepatocytes (Chieco *et al.*, 1997; Benz *et*

al., 1998; Sodeman *et al.*, 2000; Higuchi and Gores, 2003; Higuchi *et al.*, 2003; Borgognone *et al.*, 2005). In addition, it has been shown that cholestatic bile acids induce mitochondrial depolarisation (Rolo *et al.*, 2000; Huang *et al.*, 2003; Criddle *et al.*, 2004; Voronina *et al.*, 2004).

In contrast with the deleterious effects of cholestatic bile acids, it has been described that choleretic bile acids enhance bile flow (Boyer, 2002a). Furthermore, it has been reported that choleretic bile acids TUDCA and UDCA have been used at pharmacological doses to treat cholestasis (Bouchard *et al.*, 1993; Jacquemin *et al.*, 1993; Azer *et al.*, 1995; van de Meeberg *et al.*, 1996; Kinbara *et al.*, 1997; Poupon *et al.*, 1997; Beuers *et al.*, 1998; Ono *et al.*, 1998; Fabris *et al.*, 1999; Pares *et al.*, 2000; Lazaridis *et al.*, 2001).

Central to the physiological, pathological, and pharmacological actions of bile acids on hepatocytes are the bile acid-induced changes in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). The results of experiments conducted with hepatocytes and hepatocyte cell lines have shown that bile acids increase $[Ca^{2+}]_{cyt}$ (Combettes *et al.*, 1988b; Combettes *et al.*, 1990; Beuers *et al.*, 1993a; Bouscarel *et al.*, 1993), release Ca^{2+} from intracellular stores (Combettes *et al.*, 1988b, a; Beuers *et al.*, 1993a) and induce Ca^{2+} entry into hepatocytes (Beuers et al., 1993a; Beuers *et al.*, 1993b). It has been shown that while cholestatic bile acid TLCA inhibits Ca^{2+} entry (Combettes *et al.*, 1990; Beuers *et al.*, 1993a; Beuers *et al.*, 1999), choleretic bile acid (TUDCA) stimulates bile flow probably by activating Ca^{2+} entry and increasing hepatocyte $[Ca^{2+}]_{cyt}$ (Combettes *et al.*, 1988b; Beuers *et al.*, 1993a; Beuers *et al.*, 1993b; Bouscarel *et al.*, 1993). This, in turn, may activate Ca^{2+}-dependent myosin light chain kinase and the polymerisation of F-actin, and enhance contraction of the bile canaliculus (Watanabe and Phillips, 1984).

Little is known about the Ca^{2+} regulation pathways modulated by bile acids in liver cells. The next section will present some information about the regulation of Ca^{2+} homeostasis in liver cells.

1.2 Regulation of calcium homeostasis in liver cells

1.2.1. General features

An increase in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is a ubiquitous signalling messenger that regulates a broad spectrum of distinct cellular processes (Berridge, 1993; Carafoli, 2002; Parekh and Putney, 2005). In hepatocyte, $[Ca^{2+}]_{cyt}$ regulates glucose and fatty acid metabolism, bile acid secretion, protein synthesis and secretion, the movement of lysosomes and other vesicles, the cell cycle and cell proliferation, as well as apoptosis and necrosis (Leite and Nathanson, 2001; Boyer, 2002b; Enfissi *et al.*, 2004; Dixon *et al.*, 2005; Nieuwenhuijs *et al.*, 2006; O'Brien *et al.*, 2007).

An increase in cytoplasmic Ca^{2+} concentration can occur in one of two ways: through the release of Ca^{2+} from intracellular stores; or by Ca^{2+} entry across the plasma membrane. In Fig. 1.4 the different entities that contribute to the cellular cytoplasmic Ca^{2+} concentration are schematically shown.

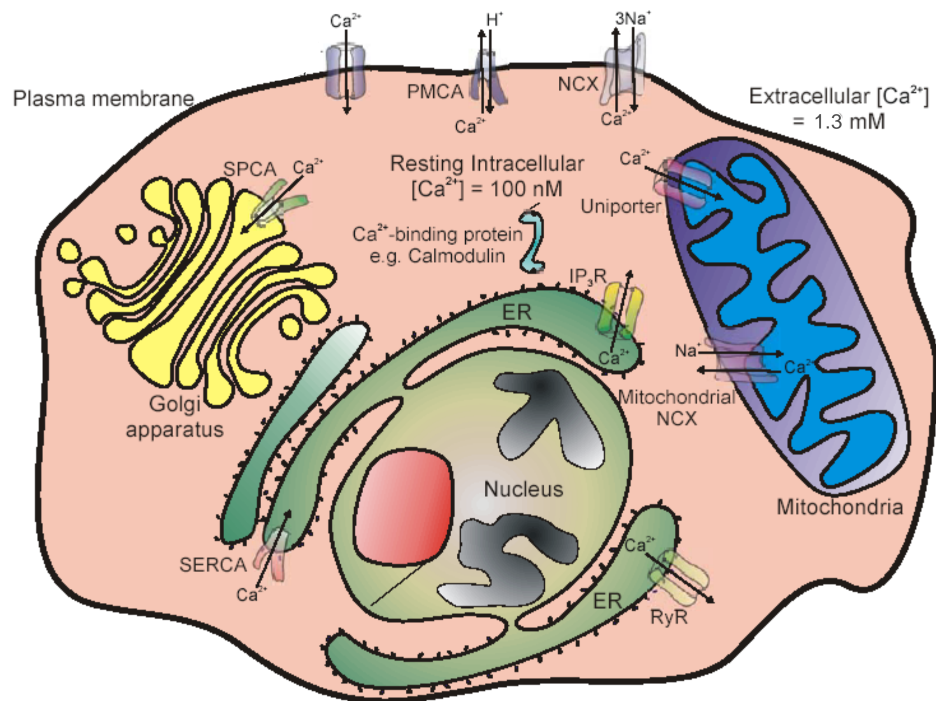

Fig. 1.4. Intracellular Ca^{2+} regulation. The plasma membrane Ca^{2+} entry channels, the Ca^{2+} extrusion across the plasma membrane mediated by the plasma membrane ($Ca^{2+} + Mg^{2+}$)ATP-ases (PMCA), and by the Na^{+}-Ca^{2+} exchanger (NCX). Ca^{2+} uptake by the ER is mediated by the ER ($Ca^{2+} + Mg^{2+}$)ATP-ase (SERCA) and Ca^{2+} outflow from the ER by IP_3 and ryanodine receptors (RyR). Ca^{2+} uptake by mitochondria is mediated by an electrogenic Ca^{2+} uni-porter and Ca^{2+} outflow by Na^{+}/Ca^{2+} and H^{+}/Ca^{2+} anti-porters. Golgi also possess IP_3R and ($Ca^{2+} + Mg^{2+}$)ATP-ase (SPCA). Numerous Ca^{2+} binding proteins are present in the cytoplasmic space and in organelles.

1.2.2. Role of intracellular stores in the regulation $[Ca^{2+}]_{cyt}$

The resting level of Ca^{2+} in the cytoplasmic space of non-excitable cells is ~ 100nM. As showed in Fig 1.4, there are several intracellular organelles such as endoplasmic reticulum, mitochondria, Golgi apparatus, lysosomes, nuclear envelope (contiguous with the ER), and possibly secretory granules, which contributes to regulated $[Ca^{2+}]_{cyt}$ (Prakriya and Lewis, 2001; Sorrentino and Rizzuto, 2001).

Among the different intracellular stores mentioned above, the endoplasmic reticulum (ER) plays a central role in the regulation of $[Ca^{2+}]_{cyt}$. Studies with hepatocytes have shown that Ca^{2+} can be released from the ER by types 1 and 2 inositol 1,4,5-trisphosphate receptors (IP_3R), (Wojcikiewicz, 1995; Hirata *et al.*, 2002) and from ryanodine receptors (Pierobon *et al.*, 2006). However, the uptake of Ca^{2+} by the ER is mediated by the ER (Ca^{2+} + Mg^{2+})ATP-ase pump (SERCA).

It has been described that the release of Ca^{2+} from the ER is involved in different cellular process, such as: hormone-induced Ca^{2+} oscillations (Woods *et al.*, 1986, 1987; Rooney *et al.*, 1989, 1990; Thomas *et al.*, 1991); vesicle trafficking (Gorelick and Shugrue, 2001); release of stress signals (Kaufman, 1999); regulation of cholesterol metabolism (Brown and Goldstein, 1999), and apoptosis (Ferri and Kroemer, 2001). The release of Ca^{2+} from intracellular stores (ER) is transient event. This is in part due to Ca^{2+}- and/or ligand-dependent inactivation of the release channels themselves; as well as due to the clearance of Ca^{2+} from the cytoplasmic space by re-sequestration into the ER (by the action of SERCA pumps) and into other organelles, particularly mitochondria (Barritt *et al.*, 2000; Gaspers and Thomas, 2005; Delgado-Coello *et al.*, 2006). Additionally, Ca^{2+} can be actively extruded from the cell by plasma membrane (Ca^{2+} + Mg^{2+})ATP-ases (PMCA) (Howard *et al.*, 1994;

Delgado-Coello *et al.*, 2003) and Na^{+}/Ca^{2+} exchangers (Studer and Borle, 1992; Gasbarrini *et al.*, 1993; Delgado-Coello *et al.*, 2006).

1.2.3. Ca^{2+} entry across the plasma membrane

1.2.3.1 Gradient of Ca^{2+} in the cells

As illustrated in Fig 1.4, the extracellular free Ca^{2+} concentration in the physiological range is ~ 1.3mM (reviewed in (Clapham, 1995)). Considering the low levels of cytoplasmic Ca^{2+} (100nM), the gradient for Ca^{2+} across the plasma membrane represents a difference in concentration of 10,000-fold. Additionally, the resting membrane potential of non-excitable cells is ~ -70mV. Despite this huge electrochemical driving force (in favour of Ca^{2+} entry), resting cells generally have low membrane permeability to Ca^{2+}. However, modest increases in the membrane permeability result in large Ca^{2+} entry, due to the Ca^{2+} electrochemical driving force. An increase in membrane permeability to Ca^{2+} can be produced by the opening of Ca^{2+}-permeable ion channels in the plasma membrane.

1.2.3.2 Ca^{2+}-permeable channels in liver cells

A variety of different Ca^{2+}-permeable channels have been found to coexist in the plasma membrane of liver cells. They are described as follows:

- Receptor-operated channels (reviewed in (Barritt, 1999; Barritt *et al.*, 2008)): these channels open as result of the binding of an agonist (such as hormones or other agonists) to its receptor. In this case, the receptor protein is separate from the channel protein. The binding of the agonist to its receptor in the extracellular space, either activates the channel directly or generates an intracellular messenger which binds to a region on the cytoplasmic domain of the channel, leading to activation of the channel.

- Ligand-gated channels ((Capiod, 1998) and reviewed in (Barritt, 1999; Barritt *et al.*, 2008)): these channels are activated directly by the binding of an external ligand to the channel proteins that contain both, the binding site for the ligand and the pore of the ion channel. They are non-selective cation channels, which, under physiological conditions, admit considerable amounts of Na^+ as well as Ca^{2+}.
- Stretch-activated Ca^{2+}-permeable channels (Bear, 1990; Bear and Li, 1991; Barros *et al.*, 2001): these channels open by transduction of mechanical forces (*eg*., plasma membrane stretch induced by increases in cellular volume).
- Store-operated Ca^{2+} channels (Rychkov *et al.*, 2001; Litjens *et al.*, 2004): these channels open in response to a decrease in the endoplasmic reticulum Ca^{2+} concentrations. They are high selective for Ca^{2+} and their main function is the replenishment of the ER Ca^{2+} levels. Moreover, it has been suggested that SOCs are the main Ca^{2+} entry pathway activated by hormones and growth factors in hepatocytes (Rychkov *et al.*, 2001; Rychkov *et al.*, 2005). This channel will be the focus of attention in the next section.

1.3 Store-operated Ca^{2+} channels (SOCs)

The concept of store-operated Ca^{2+} entry arose from the work of Putney and colleagues (Putney, 1986). They concluded that the decrease of Ca^{2+} from intracellular stores (the ER) trigged by receptor agonists, could initiate the activation of Ca^{2+} entry through the plasma membrane. Such a phenomenon was subsequently described to be a ubiquitous pathway of Ca^{2+} entry, apparently existing in all eukaryotes from yeast (Locke *et al.*, 2000) to humans (Partiseti *et al.*, 1994).

1.3.1. Physiological significance of Ca^{2+} entry through SOCs

It has been described that SOCs appears to be important for a wide range of biological processes such as: gene transcription, cell proliferation, exocytosis, secretion, chemotaxis, fertilization, among others (Fanger *et al.*, 1995; Artalejo *et al.*, 1998; Fomina and Nowycky, 1999; Saunders *et al.*, 2002; Sweeney *et al.*, 2002; Ishikawa *et al.*, 2003; Yoshida *et al.*, 2003). Additionally, it has been proposed that Ca^{2+} entry through SOCs is required for the maintenance of bile flow in rats. The mechanism proposed by which SOCs could regulated the bile flow is by contributing to the increase in the hepatocyte $[Ca^{2+}]_{cyt}$ that initiates contraction of the bile canaliculus (Gregory *et al.*, 2004a).

At the cellular level, when Ca^{2+} is released from intracellular stores, store-operated calcium entry is required as described below:

- During transient stimulation, for example occurring with cholinergic neurotransmission, Ca^{2+} entry through SOCs, replenishes rapidly the intracellular Ca^{2+} stores, leaving the cells ready for subsequent stimulations.
- Store-operated calcium entry provides a way for inducing sustained elevation in $[Ca^{2+}]_{cyt}$. For example, in the maintenance of smooth muscle tone, a sustained $[Ca^{2+}]_{cyt}$ elevation is needed. This cannot be achieved without a calcium entry mechanism, because intracellular stores contain a finite amount of Ca^{2+} to provide an elevated $[Ca^{2+}]_{cyt}$ for only few minutes.
- Finally, SOCs are described to be involved during the Ca^{2+} oscillations response. In this case, store-operated Ca^{2+} entry provides the refilling of intracellular Ca^{2+} stores, to maintain the amplitude of each intracellular Ca^{2+} spike. In the absence of Ca^{2+} entry the spike height would gradually diminish. Previous studies, with

primary cultured rat hepatocytes, demonstrated that Ca^{2+} entry through SOCs were required to maintained the oscillatory response of hormone-induced Ca^{2+} oscillations (Gregory and Barritt, 2003).

Additionally, it has been found, that Ca^{2+} entry through SOCs is involved in the regulation of several enzymes; such is the case of adenylate cyclase (Cooper *et al.*, 1998; Fagan *et al.*, 1998; Gu and Cooper, 2000) and endothelial nitric-oxide synthase (Lin *et al.*, 2000). SOCs activation may also be important for the remodelling of plasma membrane such as phosphatidyl serine externalisation, which is one of the early characteristics of cells undergoing apoptosis (Martinez *et al.*, 1999; Kunzelmann-Marche *et al.*, 2001).

1.3.2. Approaches to activate SOCs

Under physiological conditions, SOCs are activated by Ca^{2+} release from the endoplasmic reticulum induced by an increase in the levels of inositol 1,4,5-trisphosphate (IP_3), produced through receptor stimulation, or other Ca^{2+}-releasing signals (reviewed in (Parekh and Penner, 1997; Parekh and Putney, 2005)). Additionally, SOCs can be activated by any method that induces the decrease of Ca^{2+} in the ER such as:

- By addition of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors like thapsigargin and di-*tert*-butylhydroquinone (DBHQ). The inhibition of the SERCA pump would impede the active endoplasmic reticulum Ca^{2+} refilling; hence would causing the depletion of the ER Ca^{2+} content (Hoth and Penner, 1992; Parekh and Penner, 1995, 1997; Hofer *et al.*, 1998; Lewis, 1999).
- By cytoplasmic IP_3 elevation via dialysis of the cytoplasm with IP_3 (Putney, 1986; Hoth and Penner, 1992; Parekh and Penner, 1997; Rychkov *et al.*, 2001). Usually

the IP_3 addition is combined with cytoplasmic Ca^{2+} chelation induced with high concentrations of Ethylene Glycol-bis(b-aminoethyl ether)-*N,N,N´N´*-Tetraacetic Acid (EGTA). This compound chelates the Ca^{2+} that leaks from the stores and hence prevents store refilling.

- By diminishing the free intraluminal Ca^{2+} concentration in the ER via loading the cells with membrane-permeable metal Ca^{2+} chelators like *N*,*N*,*N*',*N*'-tetrakis(2-pyridylmethyl) ethylene diamine (TPEN) (Hofer *et al.*, 1998; Chan *et al.*, 2004); or by addition of Ca^{2+} ionophore ionomycin, which permeabilise the ER membrane (Morgan and Jacob, 1994).

1.3.3. Mechanism of activation of SOCs

1.3.3.1 Molecular identities of the ER Ca^{2+} sensor and the PM Ca^{2+} permeable channel

The molecular identity of the ER Ca^{2+} sensor that detects the decrease in ER Ca^{2+}, and the Ca^{2+}-permeable channel (SOCs) that consequently opens at the PM, remained elusive for almost 20 years. Recent studies have shown that Orai1 proteins constitute the pore of SOCs; while Stromal Interaction Molecule 1 (STIM1) proteins constitute the "Ca^{2+} sensor", which detects the decrease in Ca^{2+} in the ER and communicates this information to Orai1 in the plasma membrane (Liou *et al.*, 2005; Roos *et al.*, 2005; Zhang *et al.*, 2005; Feske *et al.*, 2006; Mercer *et al.*, 2006; Peinelt *et al.*, 2006; Prakriya *et al.*, 2006; Soboloff *et al.*, 2006b; Spassova *et al.*, 2006; Vig *et al.*, 2006; Yeromin *et al.*, 2006; Zhang *et al.*, 2006).

STIM1 is a protein with only one transmembrane domain and no known catalytic activity. The N-terminal region of STIM1 (within the ER lumen) includes a sterile α-motif (SAM), which often mediate oligomerization, and most importantly, a single EF-hand that binds Ca^{2+} (Williams *et al.*, 2001) (Fig. 1.5). Orai1 is a protein with

four membrane-spanning regions (M1–M4); it is incorporated into the PM with its M3–M4 loop in the extracellular space (Prakriya *et al.*, 2006) and its N and C termini in the cytoplasmic space (Feske *et al.*, 2006; Vig *et al.*, 2006) (Fig. 1.5). Orai1 mRNA is widely expressed (Feske *et al.*, 2006) and its function conserved across diverse species. The M1-M2 loop of the Orai1 channel is the region involved in the ion selectivity and Gd^{3+} inhibition (Yeromin *et al.*, 2006). Both proteins contain cytoplasmic proline-rich regions (Fig. 1.5, S/P) and coiled-coil domains (Fig. 1.5, CC), each of which could mediate protein–protein interactions (reviewed in (Taylor, 2006)).

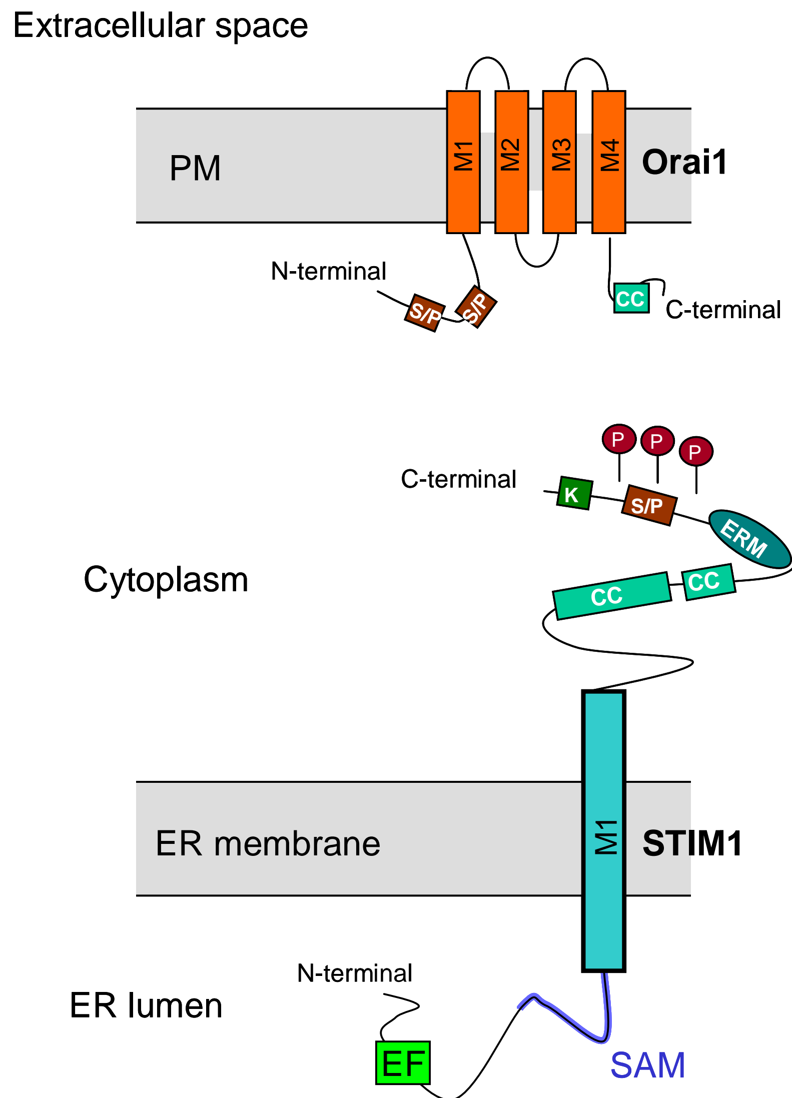

Fig. 1.5. Expected topology of STIM1 and Orai 1 proteins. STIM1 is localized primarily in the ER membrane. The organization of the major predicted domains is shown, including an unpaired EF hand and sterile-α motif (SAM) domains on the luminal side. In the cytoplasmic space side, overlapping coiled-coil (CC) and ezrin-radixin-moesin (ERM) domains, and serine-proline-rich (S/P), which includes many phosphorylation sites (P), and lysine-rich (K) domains, are shown. Orai1 located at the PM has four membrane-spanning regions and intracellular N and C terminal.

1.3.3.2 Mechanism of activation of SOCs

It has been suggested that the mechanism of activation of SOCs (Orai1) involves the sequence of events schematically shown in Fig. 1.6. These are described as follows: In resting cells with the Ca^{2+} stores full (Fig. 1.6 A), STIM1 and Orai1 are homogeneously dispersed throughout the ER and plasma membrane, respectively. When Ca^{2+} is released from the ER, the EF-hand portion of STIM1 proteins sense the decrease of Ca^{2+} inside the ER. As a consequence, STIM1 aggregates at locations where the ER is located in the vecinity of the plasma membrane ('junctional ER') (blue arrows); additionally the ER increases the number of these close contacts with the plasma membrane (red arrow) (Wu *et al.*, 2006). Moreover, Orai1 moves within the PM (green arrows) and concentrates above STIM1 aggregates. Finally, after this sequence of events, Orai1 opens allowing Ca^{2+} entry into the cytoplasmic space (Fig. 1.6 C). Subsequently, the SERCA pumps restore the ER Ca^{2+} content (Fig. 1.6 C). (Liou *et al.*, 2005; Zhang *et al.*, 2005; Baba *et al.*, 2006; Mercer *et al.*, 2006; Wu *et al.*, 2006; Xu *et al.*, 2006; Liou *et al.*, 2007; Ong *et al.*, 2007a).

It has been reported that, the peripheral ER membrane frequently comes within 8–20 nm of the PM, with which it appears to form 'junctions'. These junctions have being reported in liver cells (Guillemette *et al.*, 1988; Rossier *et al.*, 1991) and other cellular types such as excitable cells (Henkart *et al.*, 1976; Watanabe and Burnstock, 1976; Chadborn *et al.*, 2002) and yeast (Pichler *et al.*, 2001). The idea that the close relationship between the ER and the PM is required, for the activation of SOCs, has previously been suggested (Patterson *et al.*, 1999; Yao *et al.*, 1999; Rosado *et al.*, 2000; Wang *et al.*, 2000; Venkatachalam *et al.*, 2002).

Furthermore, it has been demonstrated that STIM1 redistribution is necessary for SOCs activation in lymphocytes, mast cells and other cell types (Liou *et al.*, 2005; Zhang *et al.*, 2005; Baba *et al.*, 2006; Luik *et al.*, 2006; Mercer *et al.*, 2006; Soboloff *et al.*, 2006a; Wu *et al.*, 2006; Xu *et al.*, 2006), including liver cells (Litjens *et al.*, 2007). Additionally, two independent groups have indicated that mutations within the putative pore regions of Orai1 change the biophysical properties of SOC (Prakriya *et al.*, 2006; Yeromin *et al.*, 2006).

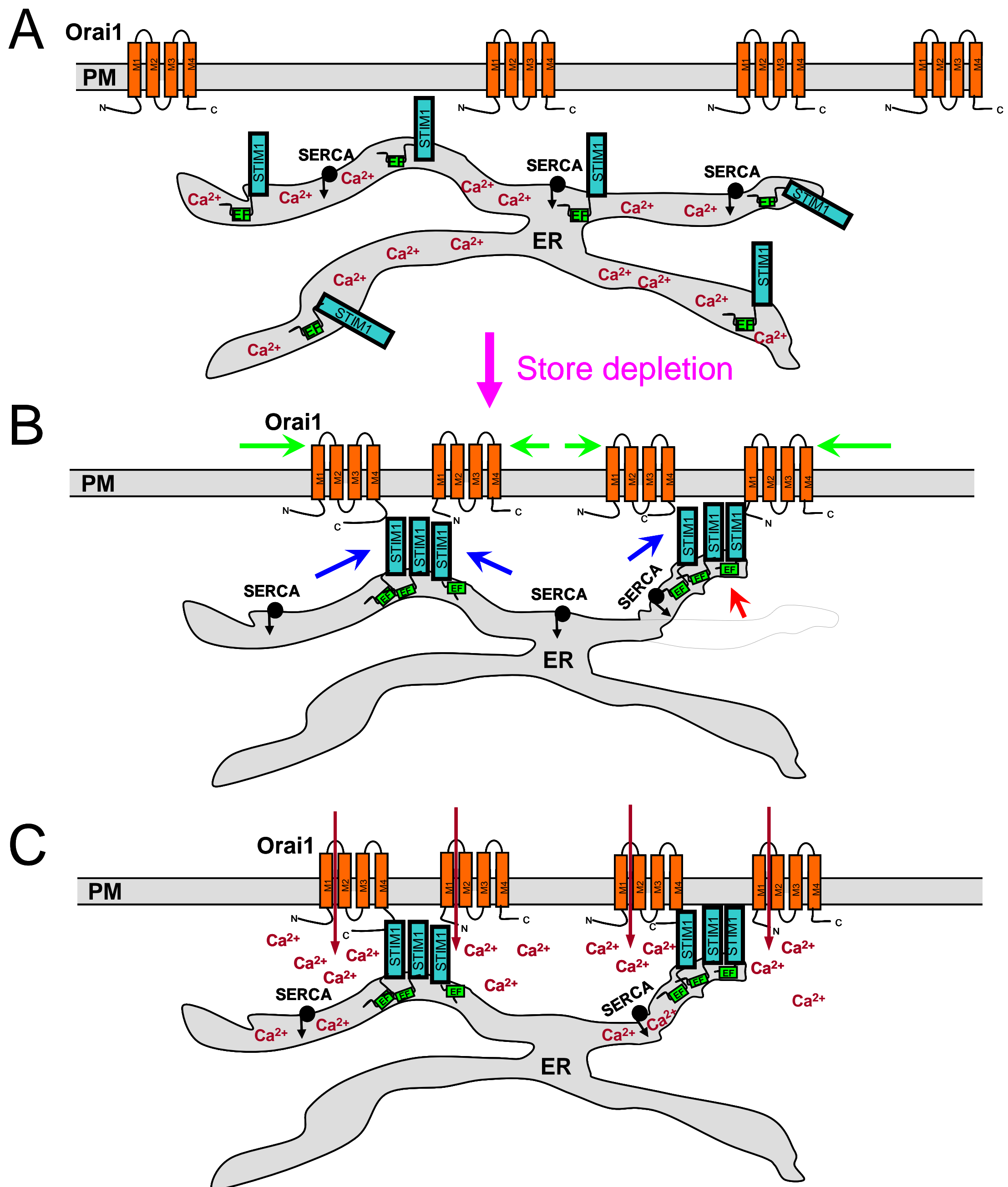

Fig. 1.6. Proposed model for the mechanism of activation of SOCs. Ca^{2+} depletion of the ER induced the redistribution of homogeneously distributed STIM1 proteins (**A**) into punctate aggregates within the ER (**B**, blue arrows). This redistribution occurs in ER regions located in close apposition to the plasma membrane and induces the activation of SOCs (CRAC). Additionally, Orai1 channels moves within the PM (**B**, green arrows) towards the STIM1 aggregates. (**C**) Once Orai1 channels at the PM activates, Ca^{2+} entry into the cytoplasmic space and the ER SERCA pumps replenish the ER Ca^{2+} content.

1.3.4. Measurement of Ca^{2+} entry through SOCs

1.3.4.1 The use of fluorescent Ca^{2+} dyes to measure Ca^{2+} entry through SOCs

A valuable and important tool for the study of SOCs activation is the use of fluorescent Ca^{2+} dyes (like fura-2). Using this method, Ca^{2+} entry can be measured; additionally Ca^{2+} release from the intracellular stores (ER) can be determined, a key step in the activation of SOCs.

Roger Y. Tsien designed the first calcium-binding indicator, called "quin2" (Tsien, 1980, 1981). The measurement of Ca^{2+} using calcium indicators is a non-invasive method in which the cells are loaded with the dye in its acetoxymethylester form. Once the dye is inside the cell, cytoplasmic esterases cleave off the ester groups and the dye remains free in the cytoplasmic space, where reports the changes in calcium concentration with great sensitivity. Nevertheless this method has some particularities as below discussed:

The Ca^{2+} dyes (like fura-2) measure the changes in the cytoplasmic Ca^{2+} concentration, which is not an accurate indication of Ca^{2+} entry across the plasma membrane. The concentration of cytoplasmic Ca^{2+} is a balance between Ca^{2+} entry and Ca^{2+} removal from the cytoplasmic space; so if Ca^{2+} entry stays constant, the net size of the cytoplasmic signal could be underestimated, because of the active removal of Ca^{2+} from the cytoplasmic space.

Additionally, in experiments conducted with Ca^{2+} dyes, the membrane potential is not controlled. SOCs are considered voltage-independent channels in the sense that they are not gated by membrane voltage changes (Hoth and Penner, 1993; Zweifach and Lewis, 1993). Depolarisation of the plasma membrane could inhibit Ca^{2+} entry by reducing the driving force for Ca^{2+} entry (Kagaya *et al.*, 2002), whereas

hyperpolarisation can reduce the size of the SOCs current, at least in RBL-1 cells (Bakowski and Parekh, 2000, 2002). Although, it is has been reported that the effect of any altered membrane potential on Ca^{2+} entry through SOCs is likely to be small (Parekh and Putney, 2005).

Finally, it is possible that the protocol used to activate SOCs, activates in addition, another Ca^{2+}-permeable channel. In this case the resulting increases in $[Ca^{2+}]_{cyt}$ would not be through SOCs channels exclusively. It has been described that thapsigargin-induced Ca^{2+} release can activate the Ca^{2+}/calmodulin-dependent protein kinase. Once active, this kinase can phosphorylate (activate) Ca^{2+}-dependent channels located at the plasma membrane (Braun and Schulman, 1995).

1.3.4.2 The use of electrophysiology to measure Ca^{2+} entry through SOCs

The other method to measure the Ca^{2+} entry through SOCs is the use of electrophysiology patch-clamp techniques to monitor the store-operated Ca^{2+} current. This technique was invented by Erwin Neher and Bert Sakmann (Neher and Sakmann, 1976). For this method, an electrode is introduced into a tiny glass pipette (filled with a saline controlled solution), which is placed against the cell membrane. The movement of ions in or out of the ion channels located in the plasma membrane can be detected by this electrode, which transmits the signal to an amplifier.

The key problem of this technique is presented by the dialysis of the cytoplasmic space via the patch pipette. Because of this, cytoplasmic factors diffuse into the pipette and are hence lost from the cell, moreover, isolation of SOCs currents requires inhibition of other currents, and hence, the ionic composition of the pipette solution is non-physiological. Finally, in some cell types, the store-operated currents

are very small, limiting the measurement of direct currents (Parekh and Putney, 2005).

Using the patch clamp electrophysiological technique in mast cells, the Ca^{2+} current induced by intracellular stores depletion was identified. The current was named Ca^{2+} Release-Activated Ca^{2+} Current (I_{crac}) and the channel responsible was called Ca^{2+} Release-Activated Ca^{2+} Channel (CRAC) (Hoth and Penner, 1992). I_{CRAC} is not the only store-operated current but remains the most used model for studying store-operated entry.

The combination of both techniques (fluorescent Ca^{2+} dyes and electrophysiology) it is considered the best strategy to study the activation of SOCs. Previous evidence obtained from our group (using patch clamp recording and fura-2 to measure $[Ca^{2+}]_{cyt}$) indicates that there is only one type of SOC in liver cells; which has a high selectivity for Ca^{2+} and its properties are essentially indistinguishable from those of Ca^{2+} release-activated Ca^{2+} channels (Rychkov *et al.*, 2001; Rychkov *et al.*, 2005).

The experiments performed and described in this thesis were done using fura-2, however a series of patch clamp complementary experiments (used in the discussion of this thesis), were undertaken in parallel by our collaborators.

1.3.5. Ca^{2+} feed back and pharmacological inhibition of SOCs

1.3.5.1. Ca^{2+} feed back inhibition of SOCs

When SOCs opens, the Ca^{2+} entry through the channel creates a micro-domain of elevated Ca^{2+} in the vicinity of each open channel, which causes the inactivation of SOCs (Hoth and Penner, 1993; Zweifach and Lewis, 1995; Fierro and Parekh, 1999). However, it has been demonstrated that, under physiological conditions, the

polarised mitochondria located below the plasma membrane can buffer the Ca^{2+} entry through SOCs. This bypasses SOCs inhibition caused by Ca^{2+} accumulation at the channel mouth, resulting in a sustained Ca^{2+} entry through SOCs. Accordingly, it has been reported that mitochondrial depolarisation inhibits Ca^{2+} entry through CRAC channels in lymphocytes and mast cells (Gilabert and Parekh, 2000b; Hoth *et al.*, 2000; Gilabert *et al.*, 2001; Glitsch *et al.*, 2002a; Malli *et al.*, 2003; Montalvo *et al.*, 2006).

1.3.5.2. Pharmacological inhibition of SOCs

Ca^{2+} entry through SOCs can be pharmacologically inhibited by addition of trivalent cations like Lanthanum (La^{3+}) and Gadolinium (Gd^{3+}) into the incubation medium. These compounds are particularly effective, blocking SOCs fully in the low micromolar concentration range (Hoth and Penner, 1993). In experiments employing fluorescent dyes to study Ca^{2+} entry through SOCs, Gd^{3+} is often used (Parekh and Putney, 2005).

Another pharmacological agent used to inhibit store-operated Ca^{2+} entry is 2-aminoethoxydiphenylborane (2-APB). 2-APB was first described as a membrane-permeable inhibitor of IP_3 receptor function, which rapidly inhibited thapsigargin-induced Ca^{2+} entry (Ma *et al.*, 2000). Electrophysiological experiments subsequently confirmed that 2-APB inhibited I_{CRAC} activation (Bakowski *et al.*, 2001; Prakriya and Lewis, 2001; Voets *et al.*, 2001). The inhibition of SOCs caused by 2-APB appears to be on an external site of the channel (Bakowski *et al.*, 2001; Prakriya and Lewis, 2001).

1.4 Role of an endoplasmic reticulum sub-region in the activation of SOCs

1.4.1. Role of the ER in the activation of SOCs

As mentioned in Sections 1.3.1 and 1.3.2, the decrease in Ca^{2+} in the ER is required for the activation of SOCs. However, it is not clearly established whether this involves the whole of the ER or only a small region. There are some studies that suggested that Ca^{2+} can freely move through the ER and that the whole of the ER is involved in the activation of SOCs (Mogami *et al.*, 1997; Subramanian and Meyer, 1997; Hofer *et al.*, 1998; Park *et al.*, 2000; Sedova *et al.*, 2000). Nevertheless, growing evidence indicates that functional compartmentalisation exists within the reticulum such that it's Ca^{2+}-releasing capabilities are not homogeneously distributed throughout the organelle (Pozzan *et al.*, 1994). In this respect, the results of some studies suggest that only a small component of the ER is required for activation of SOCs (Parekh and Penner, 1997; Hartmann and Verkhratsky, 1998; Huang and Putney, 1998; Gregory *et al.*, 1999; Parekh and Putney, 2005; Ong *et al.*, 2007b); accordingly, it has been suggested that Ca^{2+} movement through the ER is restricted (Golovina and Blaustein, 1997; Horne and Meyer, 1997).

1.4.1.1 Role of the ER sub-region in the activation of SOCs in non-liver cell types

The following points take into account the reported evidence from different experimental approaches, which suggest that only a sub-region of the ER is required for the activation of SOCs in non-liver cell types:

- In other study with NIH 3T3 cells, the treatment with phorbol esters, reduced the total Ca^{2+} content of the ER by ~ 50% (via activation of protein kinase C). However, this depletion of Ca^{2+} did not activate entry, nor did it affect the ability

of thapsigargin to activate entry when the remaining stores were emptied (Ribeiro and Putney, 1996).

- In a recent study, the abilities of two concentrations of thapsigargin (1.0nM and 1.0μM) were used to release Ca^{2+} from the ER and activate Ca^{2+} entry in human salivary gland cells (Ong *et al.*, 2007b). In that study it was observed that the addition of 1nM thapsigargin, despite the reduced capacity of Ca^{2+} release from the ER, was able to induce the complete activation of SOCs to the same extent as that of 1μM. In addition it was demonstrated that both concentrations of thapsigargin were able to induce the redistribution of STIM1 in a region of the ER located close to the PM.

- The results of experiments conducted with DT40B lymphocytes and ectopically-expressed STIM1-GFP indicate that while the bulk of STIM1 is located in the ER, some is localised in a sub-compartment, possibly comprised of mobile vesicles. When Ca^{2+} stores are depleted these vesicles accumulate just below the plasma membrane and are associated with SOCs activation (Baba et al., 2006).

- In another study, Liou et al (Liou et al., 2007), using TIRF microscopy combined with confocal microscopy, showed that upon Ca^{2+} release from the ER, STIM1 redistributes in a region of the ER located at 2 μm plasma membrane. Suggesting that region is the relevant component of the ER in the activation of SOCs.

- Recent studies using fluorescence quenching and electron microscopy have identified that after the redistribution of STMI1 within the ER, the distance of STIM1 from the plasma membrane was calculated to be ~ 6 nm. This ER-PM junction is the site of STIM1-Orai1 interactions (Varnai *et al.*, 2007).

- Finally, there is some evidence that SOCs can not be activated by the release of Ca^{2+} from the ER induced by activation of ectopically expressed Transient Receptor Potential cation channel, subfamily V, member 1 (TRPV1) in the ER (Turner *et al.*, 2003; Wisnoskey *et al.*, 2003).

1.4.1.2 Role of the ER sub-region in the activation of SOCs in liver cell

Evidence has been presented, which suggest that a small sub-region of the ER, enriched in type 1 IP_3Rs, is required for SOC activation in liver cells. It was observed that the microinjection of rat hepatocytes with adenophostin A (which has high affinity for IP_3Rs), induced the near-maximal activation of Ca^{2+} entry, with little detectable release of Ca^{2+} from intracellular stores (Gregory *et al.*, 1999). Additionally, when rat hepatocytes were microinjected with the monoclonal anti-type 1 IP_3R antibody (to inhibit Ca^{2+} release mediated by type 1 IP_3R), the addition of hormone or thapsigargin, despite causing normal release of Ca^{2+} from intracellular stores, failed to fully activate Ca^{2+} entry through SOCs (Gregory *et al.*, 1999).

In another study using microinjection of IP_3 analogues, it was found that the IP_3 analogue selective for IP_3R type 1, was more efficient in activating SOCs than the IP_3 analogue selective for IP_3R type 2 (Gregory *et al.*, 2004b). Moreover, it was found that type 1 IP_3R was located throughout most regions of the ER with some concentrated very close to the plasma membrane (Rossier *et al.*, 1991; Lievremont *et al.*, 1994; Lievremont *et al.*, 1996; Hirata *et al.*, 2002; Gregory *et al.*, 2004b; Hernandez *et al.*, 2007).

1.4.2. Use of TRPV1 to investigate the involvement of an small region of the ER in the activation of SOCs

The TRPV1 channel (Transient Receptor Potential cation channel, subfamily V, member 1) was first cloned by Julius and co-workers in 1997 (Caterina *et al.*, 1997).

TRPV1 is a nonselective ligand-gated cation channel that can be activated by a wide variety of exogenous and endogenous stimuli, including heat greater than 43°C, low pH, anandamide, N-vanillilnonamide, capsaicin and resiniferatoxin (RTX) (reviewed in (Szallasi and Blumberg, 1999)). TRPV1 receptors are found in the central and peripheral nervous systems and are involved in the transmission and modulation of pain (Huang *et al.*, 2002; Cui *et al.*, 2006).

Several studies have shown that TRPV1 is localised at the plasma membrane and at intracellular membranes. Moreover it has been shown that the activation of TRPV1, in addition to promoting Ca^{2+} entry across the plasma membrane, releases Ca^{2+} from intracellular stores (Olah *et al.*, 2001; Liu *et al.*, 2003; Marshall *et al.*, 2003; Turner *et al.*, 2003; Wisnoskey *et al.*, 2003; Karai *et al.*, 2004; Vos *et al.*, 2006; Thomas *et al.*, 2007). In the work performed by Turner and collaborators, the membrane-permeable agonist capsaicin, was used to activate intracellular TRPV1 channels expressed in RBL cells. In that work it was found that capsaicin released Ca^{2+} from intracellular stores that were contained within the total IP_3-sensitive stores. However, the release of the Ca^{2+} from the capsaicin-sensitive Ca^{2+} store failed to activate I_{CRAC} (Turner *et al.*, 2003). Using a similar approach, in insect Sf 9 and HEK-293 cells, and using fura-2 to monitor Ca^{2+} and Ba^{2+}, it was observed that in TRPV1-expressing cells, capsaicin was able to trigger both Ca^{2+} release and Ba^{2+} entry. However, in contrast to the store-operated pathway, capsaicin-induced Ba^{2+} entry was insensitive to La^{3+} and 2-APB. Moreover, these authors found that the capsaicin- and thapsigargin-sensitive stores overlapped considerably. Despite the considerable overlap, thapsigargin, but not TRPV1 agonists, was capable of activating store-operated Ca^{2+} entry. (Wisnoskey *et al.*, 2003). These two separated works may represent additional evidence for the idea that the component of the ER responsible

for signalling to store-operated channels is specialized and distinct from the bulk of the ER.

1.4.3. Use of FFP-18 Ca^{2+} dye to measure changes in Ca^{2+} levels at the vicinity of the plasma membrane

As mentioned above (section 1.4.1), the ER sub-region suggested to be responsible for the activation of SOCs, is located in the vicinity of the plasma membrane. Thus, measurements of Ca^{2+} changes restricted to that region represent key information to understand the role of the ER on the activation of SOCs.

The Ca^{2+} dye FFP-18, is a lipophilic analogue of fura-2 (Vorndran *et al.*, 1995), which has been successfully used to monitor rapid changes in the sub-plasma membrane Ca^{2+} levels (($Ca^{2+})_{SPM}$) in a variety of cells types (Etter et al., 1994; Etter et al., 1996; Davies and Hallett, 1998; Graier et al., 1998; Chadborn et al., 2002). FFP-18-AM incorporates into the plasma membrane and reorientates (by slow "flip-flop" diffusion) to the inner face of the plasma membrane where cytoplasmic esterases cleave off the ester groups. The Ca^{2+} sensitive FFP-18 acid generated on the inner face of the plasma membrane is unable to diffuse back. It has a 12-carbon hydrophobic tail that partitions into the cell membrane and a positively charged piperazine moiety that aids in binding to membrane phospholipids and thereby prevents the Ca^{2+}-binding portion of the dye from being pulled out of the plasma membrane (Etter *et al.*, 1996).

As mentioned above, the peripheral ER membrane frequently comes within few nanometers of the PM ('ER-PM junctions'). It is suggested that the diffusion of ions from the 'bulk' of the cytoplasm to the 'ER-PM junctions' could be markedly restricted (van Breemen *et al.*, 1995; Delmas and Brown, 2002; Golovina, 2005). Consequently, some studies reported that FFP-18 monitors changes in $(Ca^{2+})_{SPM}$

rather than the bulk of the cytoplasmic space ($[Ca^{2+}]_{cyt}$) (Davies *et al.*, 1997; Paltauf-Doburzynska *et al.*, 1998; Chadborn *et al.*, 2002).

One of those studies (Paltauf-Doburzynska *et al.*, 1998), describes the inability of the cytoplasmic Ca^{2+} dye fura-2 (distributed it in the bulk of the cytoplasm) to detected the Ca^{2+} release induced by ryanodine, in endothelial cells (in spite of the existence of ryanodine receptors in this cellular type). The lack of fura-2 detection was attributed to the active extrusion of the Ca^{2+} released by the plasma membrane Na^{+}-Ca^{2+} exchange; hence, in cells in which the Na^{+}-Ca^{2+} exchange was inhibited, fura-2 could effectively detect the ryanodine induced Ca^{2+} release. Moreover, when FFP-18 (distributed beneath the plasma membrane) was employed to measured $(Ca^{2+})_{SPM}$ changes, the addition of ryanodine caused a detectable Ca^{2+} release (Paltauf-Doburzynska *et al.*, 1998).

In this thesis, the Ca^{2+} dye FFP-18 is used, to estimate the changes in Ca^{2+} levels beneath the plasma membrane. This, together with the measurement of $[Ca^{2+}]_{cyt}$ reported by fura-2, offers a significant approach to understand the spatial location of the ER sub-region involved in the activation of SOCs.

1.5 Aims of the present study

The overall aims for this study are to investigate the effects and mechanism(s) of bile acids on SOCs activity, and to evaluate the role of the ER in the activation of SOCs in liver cells. The specific aims are as follows:

1- Evaluate the effects of cholestatic and choleretic bile acids on SOCs.

2- Test the ability of choleretic bile acids to counteract the effects induced by cholestatic bile acids.

3- Characterise the mechanism(s) of action of cholestatic and choleretic bile acids-mediated effects on SOCs.

4- Test if all the ER is required for the activation of SOCs in liver cells.

5- Evaluate the distribution and requirement of STIM1 proteins in bile acid-mediated SOC regulation.